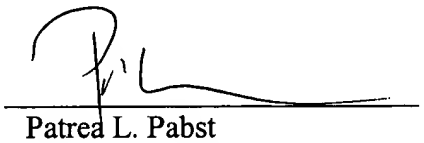
information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,



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contains *aldH* under the control of the trc promoter. *E. coli* DH5α was transformed with pMS33 or pFS14, as a negative control. Plasmid pFS14 contains the *Clostridium kluyveri 4hbD* (4HB dehydrogenase) gene, as described in Söhling and Gottschalk (1996, *J. Bacteriol.* 178:871-80).

DH5α/pMS33 and DH5α/pFS14 were grown at 37 °C with shaking in Luria-Bertani (LB; Difco; Detroit, Mich.) broth to an optical density (600 nm) of 0.5 and subsequently induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The incubation continued for 3 hours, after which the cells were removed from the medium by centrifugation (2000 *g*, 10 min.), washed in 0.1 M Tris (pH 8.0), centrifuged again, and resuspended in a volume of 0.1 M Tris (pH 8.0) roughly equal to the size of the cell pellet. Each sample was sonicated (XL sonicator, Heat Systems-Ultrasonics, Inc., Farmingdale, NY) with a microtip in 3-mL aliquots on ice for 2 min. each at a 70% cycle with a one-second interval. The lysate was spun in a microcentrifuge at 14,000 *g* for 10 min. and the supernatant was collected and designated crude cell extract.

The enzyme assays were conducted in a total volume of 1 mL containing 100 mM sodium glycine (pH 9.5), 1 mM 3-hydroxypropionaldehyde (3HPA), 1 mM NAD^+ or $NADP^+$, 6 mM dithiothreitol (DTT), and a volume of crude cell extract containing 20-100 μg total protein. A baseline was established prior to adding 3HPA, which started the reaction. The activity given by the DH5α/pFS14 extract was 0.00 U/mg when NAD^+ was used and 0.03 U/mg when $NADP^+$ was used. The activity given by the DH5α/pMS33 extract was 1.89 U/mg when NAD^+ was used and 0.32 U/mg when $NADP^+$ was used. Thus cells expressing the *E. coli* AldH protein gain the ability to convert 3HPA to 3-hydroxypropionic acid with either NAD^+ or $NADP^+$ as cofactor.

Construction of pFS14

The *4hbD* gene was cloned by PCR using the plasmid pCK3 (Söhling & Gottschalk, 1996, *J. Bacteriol.* 178:871-80) as a template. The following oligonucleotide primers were used:

5' – CTCTGAATTCAAGGAGGAAAAAATATGAAGTTATTAAAATTGGC – 3' (SEQ ID NO:1)

(*4hbD* 5' *Eco*RI)

5' – TTTCTCTGAGCTCGGGATATTTAATGATTGTAGG – 3' (SEQ ID NO:2)

(*4hbD* 3' *Sac*I)

The resulting PCR product was digested with *Eco*RI and *Sac*I and ligated to plasmid pTrcN that had been digested with the same enzymes. pTrcN is a derivative of pTrc99a (Pharmacia; Uppsala, Sweden); the modification that distinguishes pTrcN is the removal of the *Nco*I restriction site by digestion with *Nco*I, treatment with T4 DNA polymerase, and self-ligation.

Construction of pMS33

On the basis of its homology with other aldehyde dehydrogenases, the *aldH* gene was cloned by PCR from the *E. coli* genome using the following oligonucleotide primers:

5' – GGTGGTACCTTAAGAGGAGGTTTTTATGAATTTTCATCACCTGGCTT – 3' (SEQ ID NO:3)

(*aldH* 5' *Acc*65I)

5' – GGTGCGGCCGCTCAGGCCTCCAGGCTTATCCA – 3' (SEQ ID NO:4)

(*aldH* 3' *Not*I)

The resulting PCR product was digested with *Acc*65I and *Not*I and ligated to pSE380 (Invitrogen; Carlsbad, CA) that had been digested with the same enzymes to form pMS33.

Example 2: Growth of *E. coli* with 1,4-Butanediol as Sole Carbon Source

E. coli strain LS5218 (obtained from the Yale *E. coli* Genetic Stock Center, New Haven, Conn., as strain CGSC 6966) was transformed with either of two plasmids, pFS76 or pFS77. pFS76 contains the 4HB dehydrogenase (*gbd*) gene from *Ralstonia eutropha*, as described in Valentin et al. (1995, *Eur. J. Biochem.* 227:43-60). Plasmid pFS77 contains the *gbd* gene as well as the *E. coli* aldehyde dehydrogenase (*aldH*) gene and the *Klebsiella pneumoniae* 1,3-propanediol oxidoreductase (*dhaT*) gene, arranged in a single operon. Both plasmids contain the trc promoter for transcription of the genes.

LS5218/pFS76 and LS5218/pFS77 were streaked onto minimal-medium plates containing 5 g/L of either 4HB (4-hydroxybutyrate, as the sodium salt) or

1,4-butanediol. The plate medium also contained, per liter: 15 g agar; 1 mmol $MgSO_4$; 10 mg thiamine; 25.5 mmol Na_2HPO_4; 33.3 mmol K_2HPO_4; 27.2 mmol KH_2PO_4; 2.78 mg $FeSO_4 \cdot 7H_2O$; 1.98 mg $MnCl_2 \cdot 4H_2O$; 2.81 mg $CoSO_4 \cdot 7H_2O$; 0.17 mg $CuCl_2 \cdot 2H_2O$; 1.67 mg $CaCl_2 \cdot 2H_2O$; 0.29 mg $ZnSO_4 \cdot 7H_2O$; 100 μg ampicillin; and 0.1 mmol IPTG. The plates were incubated overnight at 37 °C. Both strains grew on the 4HB plate, but only LS5218/pFS77 grew on the 1,4-butanediol plate. Therefore, it was shown that the pathway consisting of the *gbd*, *aldH*, and *dhaT* genes is sufficient for growth of *E. coli* LS5218 with 1,4-butanediol as the sole carbon source.

Construction of pFS76

The *gbd* gene was amplified by PCR from the genome of *R. eutropha* H16 (obtained from the American Type Culture Collection, Rockville, Md., as strain ATCC 17699) using the following oligonucleotide primers:

5' – CCTGAATTCAGGAGGTTTTTATGGCGTTTA TCTACTATCTGACCCAC – 3' (SEQ ID NO:5)
(*gbd* 5' *Eco*RI)

5' – CCTGAGCTCCTACCTGCAAGTGCTCGCCGCTC – 3' (SEQ ID NO:6)
(*gbd* 3' *Sac*I)

The resulting PCR product was digested with *Eco*RI and *Sac*I and ligated to pSE380 (Invitrogen; Carlsbad, CA) that had been digested with the same enzymes to form pFS76.

Construction of pFS77

The *aldH-dhaT* region was removed from pMS59 by digestion with *Nhe*I and *Hin*dIII. Plasmid pFS76 was digested with *Spe*I and *Hin*dIII. *Nhe*I and *Spe*I form compatible sticky ends. The *aldH-dhaT* fragment from pMS59 and the large fragment of pFS76 were ligated together to give pFS77, containing the *gbd*, *aldH*, and *dhaT* genes, all under control of the trc promoter.

Strain LS5218/pFS30 reached an optical density (600 nm) of 3.9 and had accumulated poly-4HB to 3.3% of the dry cell weight, while strain LS5218/pMS60 reached an optical density (600 nm) of 6.5 and had accumulated poly-4HB to 12.3% of the dry cell weight. Thus expression of the *aldH* and *dhaT* genes is sufficient to increase the ability of *E. coli* LS5218 to synthesize poly-4HB from 1,4-butanediol.

Construction of pFS16

The plasmid pFS16 was constructed by ligating the *Clostridium kluyveri orfZ* (also called *hbcT*) PCR product to pTrcN. The *orfZ* gene was amplified by PCR from plasmid pCK3 (Söhling and Gottschalk, 1996, *J. Bacteriol* 178:871-80) using the following oligonucleotide primers:

5' – TCCCCTAGGATTCAGGAGGTTTTTATGGAGTGGGAA GAGATATATAAAG – 3' (SEQ ID NO:7)

(*orfZ* 5' AvrII)

5' – CCTTAAGTCGACAAATTCTAAAATCTCTTTTTAAATTC – 3' (SEQ ID NO:8)

(*orfZ* 3' *Sal*I)

The resulting PCR product was digested with *Avr*II and *Sal*I and ligated to pTrcN that had been digested with *Xba*I (which is compatible with *Avr*II) and *Sal*I to form pFS16.

Construction of pFS30

The plasmid pFS30 was derived from pFS16 by adding the *Ralstonia eutropha* PHA synthase (*phaC*) gene. The plasmid pAeT414 was digested with *Xma*I and *Stu*I so that the *R. eutropha* promoter and the structural *phaC* gene were present on one fragment. pFS16 was cut with *BamH*I, treated with T4 DNA polymerase to create blunt ends, then digested with *Xma*I. The two DNA fragments thus obtained were ligated together to form pFS30.

Construction of pMS59

The *aldH* gene was removed from pMS33 by digestion with *Spe*I and *Bgl*II. Plasmid pTC42 (Skraly et al., 1998, *Appl. Environ. Microbiol.* 64:98-105), which contains the *Klebsiella pneumoniae dhaT* gene under the control of